*Original Article*
# Circulating microRNA-423-5p serves as a potential diagnostic biomarker in elderly patients with deep-vein thrombosis

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**Abstract:** The current study aimed to investigate the diagnostic and predictive value of plasma microRNA-423-5p (miR-423-5p) for deep vein thrombosis (DVT) in elderly patients with lower extremity fractures. Eighty-four elderly patients with lower extremity fractures and 20 age- and gender-matched healthy volunteers were enrolled in this case-control study. Patients were divided into two groups, according to results of ultrasound examinations, including the DVT group and no-DVT group (patients without development of DVT during hospitalization). Levels of D-dimer and miR-423-5p in plasma were determined using immunoturbidimetry and real-time PCR (q-PCR). Compared to control (con) and no-DVT groups, plasma miR-423-5p was significantly upregulated on the first day of hospitalization in the DVT group (P < 0.05). In the DVT group, perioperative plasma miR-423-5p expression levels gradually increased from 17.86 to 29.81, then remained at a high level with no downward trend. Receiver operating characteristic curve (ROC) analysis showed that the area under the curve (AUC) of plasma miR-423-5p and D-dimer, respectively, was 0.663 (95% CI: 0.545-0.781) and 0.491 (95% CI: 0.364-0.617). Present data suggests that plasma miR-423-5p serves as a potential non-invasive biomarker for DVT in elderly patients with lower extremity fractures.

**Keywords:** Elderly, lower extremity fracture, deep vein thrombosis, d-dimer, microRNA-423, biomarker

## Introduction

Deep vein thrombosis (DVT) is one of the most common complications during the perioperative period in elderly patients with lower extremity fractures. Trauma and advanced age are important factors in the development of DVT [1, 2]. Once the emboli of the DVT patient falls off, the embolus will return to the pulmonary artery with the blood stream, resulting in the development of pulmonary embolisms (PE). In the United States, the number of PE patients has reached 600,000-900,000 per year. The death toll has reached 200,000, with most deaths occurring within 1 hour after onset [3]. Common clinical manifestations of DVT include limb swelling, erythema, and limb tenderness, which are easily covered by inflammatory reactions caused by fractures and trauma. Diagnostic methods for DVT include lower extremity venography procedures [4], lower extremity venous ultrasounds, and D-dimer. Plasma D-dimer is a biomarker of DVT. It is highly sensitive for diagnosis of DVT, but its specificity is very low [5]. Therefore, it is necessary to find new DVT biomarkers for elderly patients with lower extremity fractures.

MicroRNAs are highly conserved non-coding small single-stranded RNAs (approximately 22 bases) that bind to mRNA by base pairing [6], thereby inhibiting mRNA translation and regulating protein expression [7]. They can be stably present in the circulatory system and are resistant to harsh conditions, including RNase, temperature changes, pH changes, repeated freeze-thaw cycles, and long-term storage [8, 9]. Generally, expression levels of miRNAs are different in different cells or tissues. Expression patterns can be altered at the beginning of the pathological process. Therefore, circulating miRNAs are expected to be a novel biomarker for

DVT in elderly patients with lower extremity fractures.

To the best of our knowledge, there are no other studies concerning the relationship between DVT and circulating miRNAs in elderly patients with lower extremity fractures. Accordingly, the current study collected plasma from different time points in the perioperative period of elderly patients with lower extremity fractures. This study measured D-dimer and miR-423-5p levels of expression in plasma using immunoturbidimetry and q-PCR methods. Effects of trauma, surgery, and thrombus factors on plasma miR-423-5p and D-dimer expression levels were then analyzed, determining the accuracy of plasma miR-423-5p and D-dimer for DVT diagnosis. The conclusion was drawn that plasma miR-423-5p is superior to D-dimer in the diagnosis of DVT in elderly patients with lower extremity fractures.

## Design and methods

### Patient recruitment

Between April and July 2017, a total of 84 elderly patients with lower extremity fractures in the Department of Geriatrics Orthopedics, the 3rd Hospital of Hebei Medical University, participated in the current study. Patients had an average age of 77.65 years (standard deviation, SD, 8.18 years). They were divided into the DVT group (47 cases) and no-DVT group (37 cases), according to results of deep venous vein ultrasonography procedures of the lower extremities after admission. A total of 20 subjects participated in the control group. Inclusion criteria for the experimental group: (1) Age ≥ 65 years; (2) X-ray diagnosis of lower limb fractures; (3) Patients with lower limb fractures that intended to undergo surgery; and (4) Willing to participate in the study and provided informed consent. Exclusion criteria for the experimental group: (1) Patients with long-term thrombotic diseases; (2) Old fractures (fracture occurrence before two weeks and more); (3) Patients with severe medical diseases that could not tolerate surgery; (4) Patients that had serious complications after surgery, needing special treatment; (5) Patients with poor compliance; and (6) Patients refusing to participate in the experiment. Exclusion criteria for healthy controls: (1) Age < 65 years; (2) Recent history of trauma; (3) Patients highly dependent of others for daily activities or bed rest for a long time; and (4) Serious medical diseases. The current study was reviewed by the Ethics Committee of the 3rd Hospital of Hebei Medical University. All subjects were informed of the purpose of the experiment and provided informed consent before enrollment.

### Plasma preparation and preservation

After fasting for 8 hours, 6 mL of peripheral venous blood was drawn from the elbow vein. The blood was dispensed into two 3 mL vacuum blood collection tubes (INSEPACK, China), containing 3.28% sodium citrate. They were immediately stored in a refrigerator at 4°C. Blood samples were stored in the refrigerator at 4°C for no more than 2 hours. One sample was sent to the Department of Clinical Laboratory, the RD Hospital of Hebei Medical University, for detection of plasma D-dimer expression. The other was sent to the Clinical Laboratory of the 3rd Hospital of Hebei Medical University. After centrifugation at 1,900 × g for 10 minutes at 4°C, the supernatant (platelet-poor plasma) was transferred to a cryotube without RNase/DNase (Biologix, USA) and immediately stored in a -80°C refrigerator for detection of microRNA expression levels in plasma.

### RNA isolation

Total RNA was extracted from 200 µl of plasma using a Qiagen miRNeasy Mini Kit (QIAGEN, Germany), according to manufacturer instructions. Specific procedures were as follows. The plasma was taken out from the -80°C freezer, thawed on ice, and centrifuged at 120,000 × g for 10 minutes in a 4°C microfuge. Next, 200 µl of the supernatant was taken from the sample, transferred to a new microcentrifuge tube, and 1,000 µl of QIAzol Lysis Reagent was added to the plasma. After thorough mixing, the mixture stood at room temperature for 5 minutes. Afterward, 3.5 µl of miRNeasy Plasma Spike-In Control (1.6 × 10$^8$ copies/µl working solution) was added and mixed well. Next, 200 µl of chloroform was added, thoroughly mixed, and centrifuged at 12,000 × g for 15 minutes at 4°C. The upper aqueous phase was transferred to a new microcentrifuge tube and 900 µl of 100% ethanol was added. After thoroughly mixing, the liquid was transferred to a Qiagen RNeasy Mini spin column in a collection tube and centrifuged at 8,000 × g for 15 seconds at room tem-

perature. Buffer RWT, Buffer RPE, and 80% ethanol were then added to a Qiagen RNeasy Mini spin column to wash the column membrane. The column membrane was spin-dried at 25,000 × g for 5 minutes and 14 μl of RNase-free water was added to the column membrane and incubated for 1 minute. It was then centrifuged at 25,000 × g for 1 minute at room temperature to elute total RNA. Eluted total RNA was stored in a -80°C refrigerator.

*Quantitative real-time PCR (qPCR) assay*

Plasma miR-423-5p expression levels were determined using q-PCR. The experimental procedure was as follows. Reverse transcription of all miRNAs into cDNA was conducted using the miRNA First Strand cDNA Synthesis Kit (Vazyme, China), according to manufacturer protocol. Quantitative PCR assays were performed using the miRNA Universal SYBR PCR Master Mix kit (Vazyme, China), according to manufacturer protocol. All samples were in triplicate. To normalize results, hsa-miR-U6 was used as an internal reference gene. Primer sequences of primers miR-423-5p were designed using primer design software (CE Design V1.04) supplied by Vazyme: 5'-GTGAGGGGCAGAGAG-CGA-3' and 5'-GTCGTATCCAGTGCAGGGTCCGA-GGTATTCGCACTGGATACGACAAAGTC-3'. Primer sequences of hsa-miR-U6: 5'-CTCGCTTCGGC-AGCACA-3' and 5'-AACGCTTCACGAATTTGCGT-3'. PCR was carried out under the following cycle conditions: 95°C for 15 minutes, followed by 45 amplification cycles (denaturation at 95°C for 15 seconds, primer annealing at 55°C for 30 seconds, and extension 70°C, 30 seconds). The crossover point (CP) was used to calculate expression levels of the miRNA. When CP is greater than 36, miRNA expression levels are considered to be very low. Thus, further analysis is not required. Expression levels of miR-423-5p were scalculated using the $2^{-\Delta\Delta Cp}$ method [10].

*Plasma D-dimer detection*

To accurately determine expression levels of plasma D-dimer, blood samples were sent to the Clinical Laboratory of the Third Hospital of Hebei Medical University within 2 hours. They were measured by HemosIL D-Dimer HS (Instrumentation Laboratory, Bedford, MA, USA) on an ACL TOP® 700 LAS (Instrumentation Laboratory) device. Immunoturbidimetric as-

says were performed to guarantee the quality of D-dimer values.

*Statistical analysis*

Statistical analyses were performed using SPSS software version 21.0 (SPSS, Inc., Chicago, IL, USA). Two-tailed *p*-values less than 0.05 indicate statistical significance. Moreover, $x^2$ tests and one-way analysis of variance (ANOVA) were used to compare differences in clinical features between categorical and continuous variables. Mann-Whitney U-test was used to compare differences in miRNA levels between groups. Data are expressed as mean ± 95% confidence intervals (CI). Correlation between levels of D-dimer and miRNAs was evaluated using Pearson's correlation analysis. Receiver operating characteristic (ROC) curves of miR-423-5p were constructed, evaluating the clinical value of miR-423-5p in the diagnosis of DVT.

## Results

*Correlation of plasma miR-423-5p and DVT development*

According to results of ultrasound color Doppler imaging (**Figure 1**), elderly patients with lower extremity fractures were divided into the DVT group and no-DVT group. There were no significant differences in general clinical data between elderly patients with lower extremity fractures and healthy controls, regarding sex ratio, mean age, diabetes, and hypertension (P > 0.05) (**Table 1**). Expression levels of plasma miR-423-5p in each group on the first day of admission were detected by q-PCR. Expression levels of miR-423-5p were calculated using the $2^{-\Delta\Delta Cp}$ method. As shown in **Figure 2A**, plasma miR-423-5p expression was significantly higher in the DVT group than that in the control group and no-DVT group (P < 0.05). However, there were no significant differences in plasma miR-423-5p expression between the no-DVT group and control group (P > 0.05). Expression levels of plasma miR-423-5p in the DVT group and no-DVT group were 15.47 and 6.54-fold of plasma miR-423-5p expression levels in the control group, respectively. Expression levels of plasma miR-423-5p in the DVT group were 2.37-fold that of the no-DVT group. In the DVT group, gender, hypertension, and diabetes were found to have no statistically significant effects on
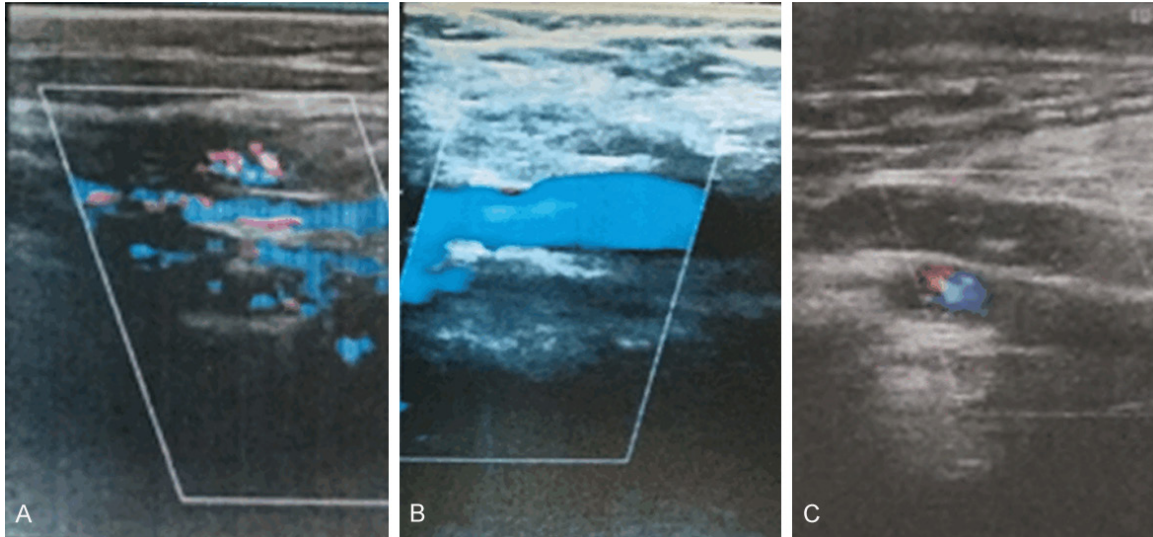
**Figure 1.** Results of deep vein color Doppler ultrasound imaging of the lower extremities in elderly patients with lower extremity fractures. Color Doppler ultrasonography was used to detect the size and location of thrombus structures. There was no significant stenosis and obstruction of the lower extremity vein in patients with no-DVT, as shown in (A). Lower extremity venous thrombosis in patients with DVT causes venous stenosis. The sagittal and coronal positions of color Doppler ultrasound imaging are shown in (B and C).

**Table 1.** General clinical data of study subjects

| Variables | Controls (20) | No-DVT patients (37) | DVT patients (47) | Total (104) |
|---|---|---|---|---|
| Ages (years) | 71.25±5.95 | 75.38±7.76 | 79.45±8.13 | 76.42±8.18 |
| Male/Female | 6/14 | 12/25 | 14/33 | 32/72 |
| Diabetes (yes/no) | 7/13 | 9/28 | 7/40 | 23/81 |
| Hypertension (yes/no) | 12/8 | 15/22 | 15/32 | 42/62 |

plasma miR-423-5p levels (**Figure 2B-D**), with P > 0.05. There were no significant differences in plasma miR-423-5p levels in terms of gender, hypertension, and diabetes in the no-DVT group (P > 0.05). Results are presented in **Figure 2E-G**. Present data indicates that expression levels of plasma miR-423-5p are significant prognostic markers for DVT development.

*Plasma miR-423-5p and D-dimer in perioperative DVT and no-DVT groups*

To further understand the effects of trauma, surgery, and thrombosis on perioperative plasma miR-423-5p and D-dimer levels in elderly patients with lower extremity fractures, their values were measured at different time points. On the 1st day after hospitalization, 1 day before surgery, 1 day after surgery, and 7 days after surgery, a total of 6 mL of fasting venous blood was collected. Levels of D-dimer and miR-423-5p in plasma were determined by im-

munoturbidimetry and q-PCR. Results are shown in **Figure 3**. Expression levels of plasma miR-423-5p in the DVT group were significantly higher than those in the no-DVT group and contol group (P < 0.05). As shown in **Figure 3A**, expression levels of plasma miR-423-5p in the no-DVT group were significantly affected by trauma and surgical strike factors. Expression levels of miR-423-5p were significantly increased after fractures and surgery, especially at 1 day after surgery. Levels were significantly different from those before surgery (P < 0.05). As trauma and surgical factors disappeared, expression levels of plasma miR-423-5p also decreased. Expression levels of miR-423-5p in the DVT group gradually increased during the perioperative period, from 17.86 on the first day after hospitalization to 29.81 on the 7th day after the operation. There was no downward trend. At each of the four perioperative blood collection points, expression levels of plasma miR-423-5p in the DVT group were higher than those in the no-DVT group. Differences between the three blood collection points were statistically significant (P < 0.05). At 1 day after surgery, expression levels of miR-423-5p in the DVT

Figure 2. Expression levels of miR-423-5p in plasma of elderly patients with lower extremity fractures and healthy individuals. The histogram (A) Shows the expression level of miR-423-5p in plasma on the first day of hospitalization, con group, no-DVT group and DVT group. Histograms (B-D) Show the effects of gender, hypertension, and diabetes on plasma miR-423-5p in patients with DVT (p > 0.05). Histograms (E-G) Show the effects of gender, hypertension, and diabetes factors on plasma miR-423-5p in the absence of DVT (P > 0.05).
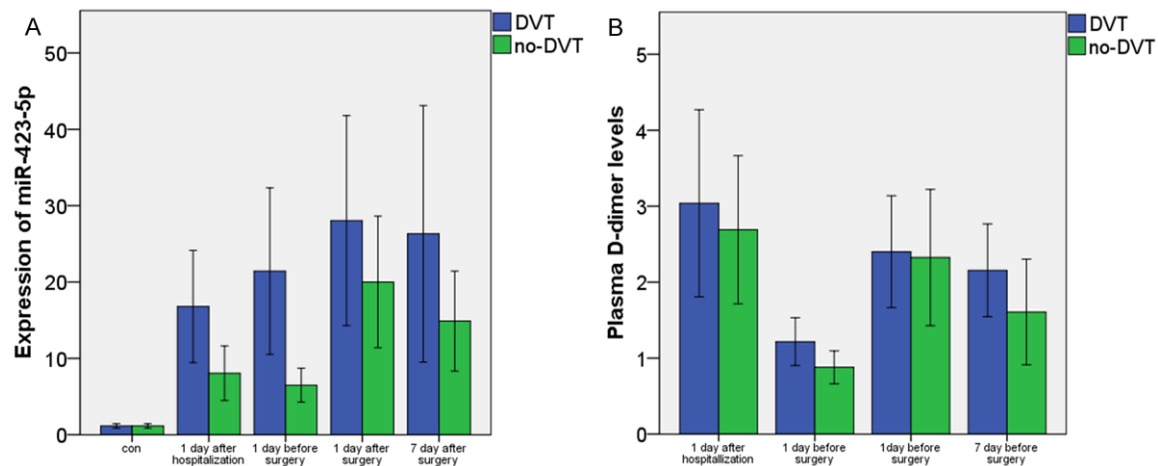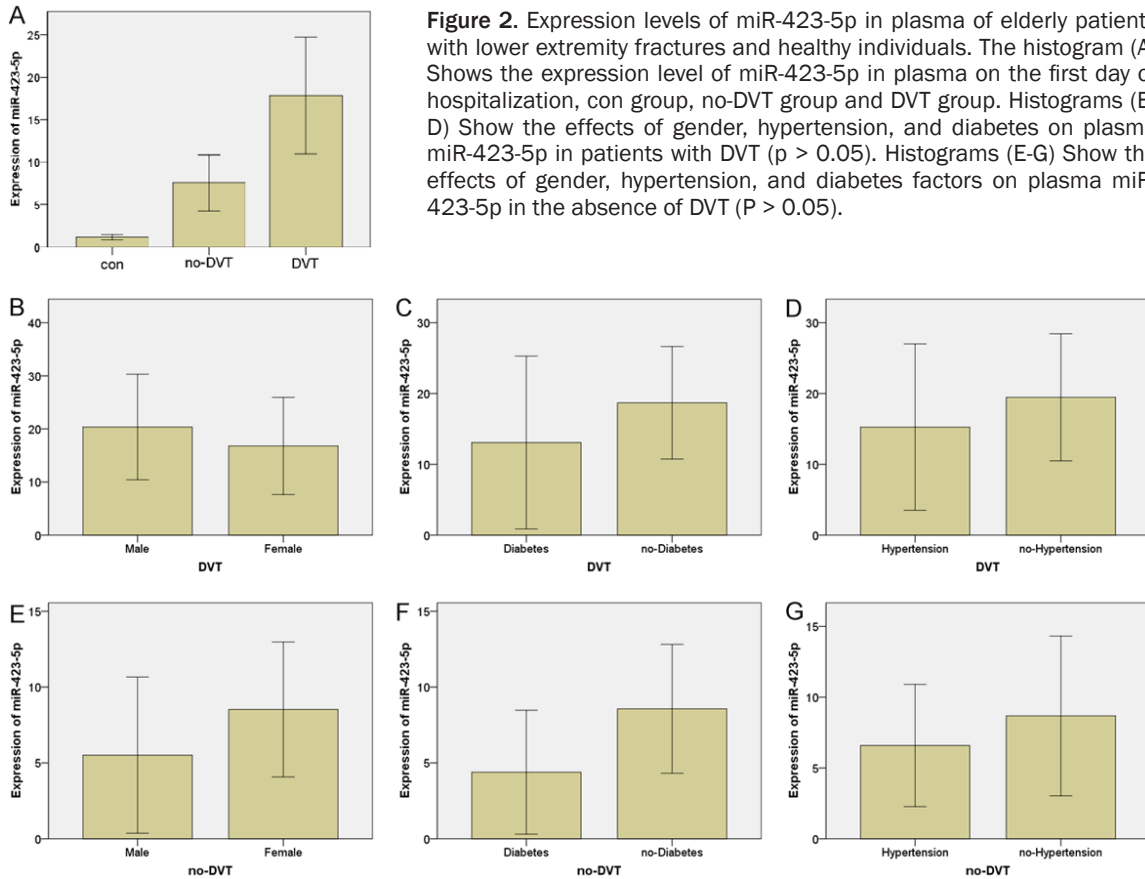


Figure 3. Trends in plasma miR-423-5p and D-dimer in the perioperative period in elderly patients with lower extremity fractures. Histogram (A): Mean expression levels of plasma miR-423-5p in elderly patients with lower extremity fractures during the perioperative period (1 day after hospitalization, 1 day before surgery, 1 day after surgery, and 7 days after surgery). Histogram (B): Mean expression level of plasma D-dimer in perioperative patients with lower extremity fractures in the elderly.

group were higher than those in the no-DVT group, but differences were not statistically significant (P > 0.05). The trend of plasma D-dimers in patients with DVT and no-DVT (**Figure**

3B) showed that plasma D-dimers responded significantly to both trauma and surgical factors (P < 0.05). There were also significant differences in plasma D-dimer expression levels
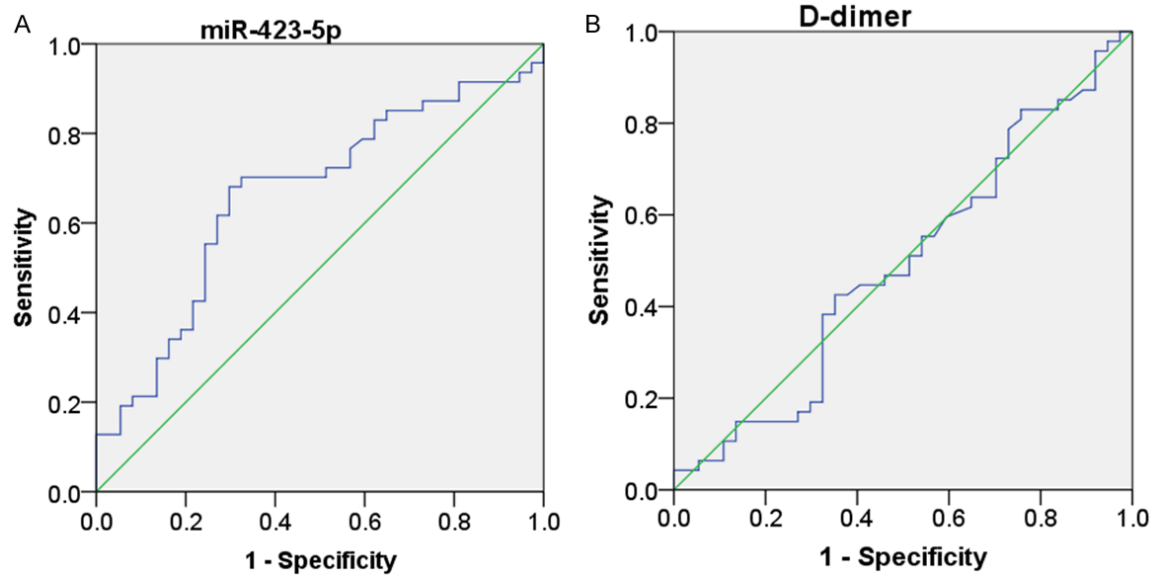
**Figure 4.** ROC curve analysis results of diagnostic and predictive value of plasma miR-423-5p and D-dimer in elderly patients with lower extremity fracture. The areas under the ROC curve for the diagnosis and predictive value of plasma miR-423-5p (A) and D-dimer for DVT (B) were 0.663 (95% CI: 0.545-0.781) and 0.491 (95% CI: 0.364-0.617), respectively.

between the two groups before and after surgery ($P < 0.05$). Expression levels of plasma D-dimer in the DVT group were higher than those in the no-DVT group, but differences were statistically significant only 1 day before surgery ($P < 0.05$).

*Diagnostic value of plasma miR-423-5p and D-dimer for DVT in elderly patients with lower extremity fractures*

Of the elderly patients with lower extremity fractures participating in this experiment, 96.5% of the patients had higher plasma D-dimer levels than the normal range (0-0.3 mg/L). Of these, 97.3% of patients in the DVT group had higher D-dimer levels than the normal range. In the no-DVT group, 95.5% of patients had D-dimer levels higher than the normal range. ROC curve analysis was performed to determine the roles of plasma miR-423-5p and D-dimer in diagnosis and prediction of DVT in elderly patients with lower extremity fractures. Results (**Figure 4A** and **4B**) showed that the area under the ROC curve (AUC) of plasma miR-423-5p and D-dimer was 0.663 (95% CI: 0.545-0.781) and 0.491 (95% CI: 0.364-0.617), indicating higher predictive value of the former, compared to the latter, in development of DVT.

*Correlation between plasma D-dimer and miR-423-5p in elderly patients with lower extremity fractures*

Investigating whether miR-423-5p expression levels correlate with plasma D-dimer levels in elderly patients with lower extremity fractures, Pearson's correlation analysis was conducted. Results showed no correlation between expression levels of plasma miR-423-5p and D-dimer in the DVT group or in the no-DVT group (**Figure 5A** and **5B**).

Discussion

Although its specificity for diagnosis of DVT in elderly patients with lower extremity fractures is very low [11], D-dimer has still been widely used in clinical practice. This is due to the lack of other biomarkers in the development of DVT. Therefore, there is an urgent need to find a new type of DVT biomarker, improving the accuracy of DVT diagnosis. In recent years, there have been many reports indicating that miRNA expression profiles are associated with the development of various diseases, such as tumors [12], cardiovascular disease [13], and immune system diseases [14]. There are also many studies concerning circulating miRNA dysregulation in patients with DVT [15-18]. Stu-
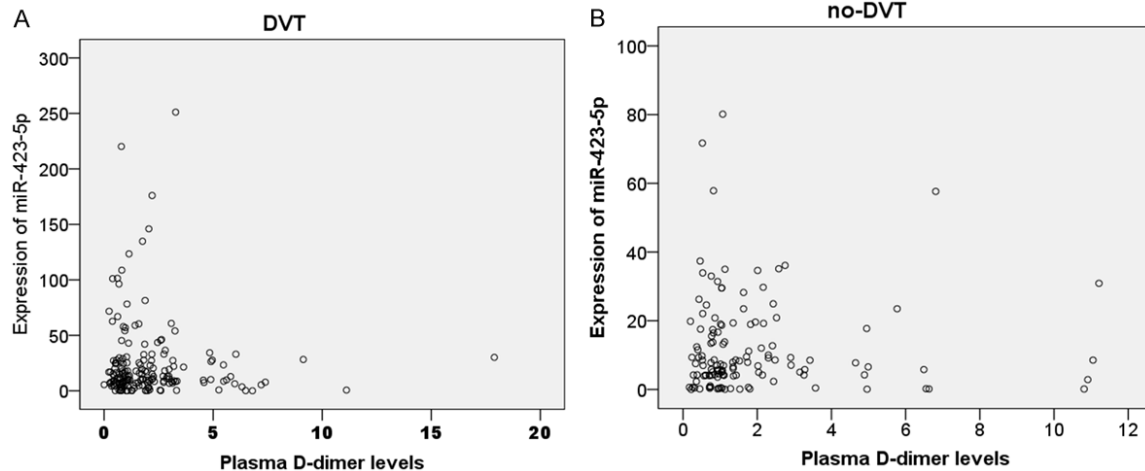
**Figure 5.** Correlation between circulating miR-423-5p and D-dimer in elderly patients with lower extremity fractures. Plasma miR-423-5p values plotted against D-dimer levels (A and B). There was no significant correlation between miR-423-5p and D-dimer in the DVT group and no-DVT group.

dies of circulating miRNAs as diagnostic criteria for diseases are highly sought after because circulating miRNAs have the following advantages: 1) Circulating miRNAs are very stable, making detection methods reproducible; and 2) Changes in circulating miRNA expression profiles are related to disease specificity. In addition, circulating miRNAs can be obtained in a non-invasive manner, having the advantages of small sampling damage and a convenient operation. This is easy for patients to accept.

The current study used q-PCR to detect expression levels of plasma miR-423-5p in elderly patients with lower extremity fractures, as well as healthy controls. Results showed that expression levels of plasma miR-423-5p in the DVT group and no-DVT group were higher than those in the control group. Expression levels of plasma miR-423-5p are elevated after fractures, possibly caused by trauma, pain and stress response. However, no significant differences were found in expression levels of plasma miR-423-5p between the no-DVT group and con group. Reasons may include: 1) The number of participants in this experiment was small; 2) Patients participating in this experiment differed in the degree of injury; and 3) Factors, such as trauma, pain, and stress response, have little effect on expression of miR-423-5p. In contrast, expression levels of plasma miR-423-5p in the DVT group were significantly higher than those in the control group and no-DVT group (P < 0.05). During hospitalization, plasma miR-423-5p remained at a high level

in the DVT group, with no downward trend. Expression levels of plasma miR-423-5p in the no-DVT group decreased with the disappearance of trauma and surgical factors. Thus, it was speculated that plasma miR-423-5p expression levels in the DVT group are higher than plasma miR-423-5p expression levels in the no-DVT group and can be maintained at high levels. This is possibly due to certain factors, including thrombosis or inflammation. In the study by Gidlöf et al., the authors observed activated platelets during thrombus releasing miR-423-5p into the bloodstream [19]. Reducing the inflammatory response, the body increases expression levels of plasma miR-423-5p [20].

ROC curve analysis showed that the area under the ROC curve (AUC) of plasma miR-423-5p and D-dimer, respectively, was 0.663 (95% CI: 0.545-0.781) and 0.491 (95% CI: 0.364-0.617). This data indicates that plasma miR-423-5p has a higher diagnostic ability in elderly patients with lower extremity fractures. When used in clinical practice, this can not only reduce the forced movement of elderly fracture patients and reduce risks of other diseases caused by thrombosis and pain, but also diagnose DVT in a timely manner. Reasons for the smaller area under the D-dimer ROC curve may include: 1) Patients participating in the experiment were older and the false positive rate of D-dimer in the elderly was significantly higher than that in the young [21]; and 2) The severity of bone trauma patients was different, with

more severe trauma indicating more obvious increases of D-dimer [22].

There were several general limitations to the current study, however. First, current results are based on a relatively small sample size, which may have biased statistical results. Second, the population targeted by this research was special, possibly leading to differences in results from other studies. Third, the current study did not examine specific reasons for the upregulation of plasma miR-423-5p expression in elderly patients with fractures. Fourth, this study focused only on a single microRNA. Since individual genes may have multiple microRNA potential targets [23], multiple microRNAs may be more reliable as disease biomarkers. Accordingly, in the future, well-designed study (randomized controlled trails) with large sample sizes are necessary, investigating more specific biomarkers and other related influential factors.

In summary, the current study investigated the diagnostic and predictive value of plasma microRNA-423-5p (miR-423-5p) for deep vein thrombosis (DVT) in elderly patients with lower extremity fractures. Results suggest plasma microRNA-423-5p as a better predictive biomarker for development of DVT. Present results could have preventive importance in the management of elderly patients with traumatic fractures.

## Disclosure of conflict of interest

None.

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